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For: METHODS AND COMPOSITIONS USING NEURAL PROGENITORS

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2. 8 sheets of drawings.

3. Please charge the required application filing fee of **$80.00** and any other fees that may be required, to the deposit account of **Kenyon & Kenyon**, deposit account number **11-0600**. A duplicate of this sheet is enclosed.

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METHODS AND COMPOSITIONS USING NEURAL PROGENITORS

FIELD OF THE INVENTION

The present invention provides methods and compositions involved in migration of undifferentiated neural progenitor cells, which can be useful in treatment of conditions associated with neurogenesis.

BACKGROUND OF THE INVENTION

Migration of immature neurons during development is essential for the proper formation of the nervous system. In the mammalian brain, most neurons are generated within proliferative zones around the ventricle from where immature precursors migrate to specific sites in the cerebral wall (Marin and Rubenstein, 2003). A variety of clinical syndromes, including various forms of Lissencephalies, are related to deficient migration of neural cells (Ross and Walsh, 2001). The consequences of these malformations include mental retardation, epilepsy, paralysis and blindness. Genetic studies of some of these perturbations have provided some understanding of the regulation of neuronal migration, which has rapidly expanded over the past ten years (Rubenstein and Rakic, 1999; Ross and Walsh, 2001).

In addition to playing a key role in early development, neuronal migration is also important for the adult brain. For example, in the brain of songbirds, neurogenesis and neuronal migration are required for structural plasticity and learning throughout adulthood (Goldman and Nottebohm, 1983). Recent evidence suggests that undifferentiated multipotential progenitors also exist in the adult mammalian brain and during adult neurogenesis, as well as during the continuous neuronal replacement that occurs at specific sites in the rostral subventricular zone-olfactory bulb system and the dentate gyrus (Alvarez-Buylla and Garcia-Verdugo, 2002; Marshall et al., 2003).

Finally, cell migration plays a central role in wound repair. Although the intrinsic capacity of the adult mammalian brain to replace lost or damaged neurons is very limited,

migration of neural progenitors and cell replacement has been reported after administration of various factors.

Considerable effort has recently been focused on understanding the factors and mechanisms involved in the navigation of immature neurons to their final destination. Highly conserved families of attractive and repulsive molecules are coordinately regulated in order to guide neurons to their final destination. These include netrins, semaphorins, ephrins, Slits and various neurotrophic factors (Marin and Rubenstein, 2003; Marin et al., 2003). Compared to migration of postmitotic immature neurons, little is known about the factors and mechanisms that direct the migration of neural stem cells and undifferentiated progenitors. In one study, placental derived growth factor (PDGF) was shown to attract FGF-2-stimulated neural progenitors in a transfilter migration assay (Forsberg-Nilsson et al., 1998).

Angiogenesis and neurogenesis have been shown to occur concurrently in the adult dentate gyrus (Palmer et al., 2000) and in the songbird brain (Louissaint et al., 2002). Recent evidence further suggests that vascular endothelial growth factor (VEGF) is expressed in neural cells and plays a role in diverse aspects of brain development, including axonal growth (Sondell et al., 1999; Sondell et al., 2000), cell survival (Sondell et al., 1999; Jin et al., 2000; Ogunshola et al., 2002) and neuroprotection against glutamate toxicity (Matsuzaki et al., 2001; Svensson et al., 2002). Moreover, it has been suggested that VEGF can enhance neurogenesis in the subventricular zone in vivo as well as in vitro (Jin et al., 2002).

VEGF belongs a family of glycoproteins that play an essential role in the development of blood vessels (vasculogenesis), the generation of new vascular networks from existing vessels (angiogenesis) and in hematopoiesis (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001). The VEGF gene family has at least five members in mammals, including VEGF-A, VEGF-B, VEGF-C and VEGF-D and placenta growth factor (PIGF) (Matsumoto and Claesson-Welsh, 2001). These secreted molecules bind with high affinity to tyrosine kinase receptors including vascular endothelial growth

factor receptor 1 (VEGFR1, also known as Flt1), vascular endothelial growth factor receptor 2 (VEGFR2, also known as KDR/Flk1), vascular endothelial growth factor receptor 3 (VEGFR3, also known as Flt-4) and to Neuropilins -1 and -2. As a result of the receptor activation and subsequent signal transduction, VEGF target cells may proliferate, migrate, or alter gene expression, e.g. of matrix metalloproteases or cytokines.

Identifying candidate molecules that could play a role in this process is crucial not only for understanding proper tissue formation during development, but also for developing methods for directing undifferentiated progenitors to achieve structural brain repair.

SUMMARY OF THE INVENTION

Migration of undifferentiated neural progenitors is critical for the development and repair of the nervous system. In the present invention, VEGF A, a major angiogenic factor, guides the directed migration of neural progenitors that do not display antigenic markers for neuron- or glia-restricted precursor cells. These progenitor cells express both VEGFR1 and VEGFR2, but signaling through VEGFR2 specifically mediates the chemotactic effect of VEGF. The expression of VEGF receptors and the chemotaxis of progenitors in response to VEGF is enhanced in the presence of FGF-2.

In the context of the present invention, VEGF functions as a factor to recruit neurogenic progenitors to specific sites and to induce directional sensing by neural progenitor cells. Chemoattraction by VEGF can be mediated by signaling through VEGFR2. In addition, FGF-2 increases expression of VEGF receptors in neural progenitors and the chemotactic response of progenitor cells occurs in the presence of FGF-2.

Thus, the present invention provides methods and compositions useful in formation of the nervous system and migration of neural progenitor cells, including treatment of conditions associated with malformations in the nervous system. These inventive methods and compositions can also be useful in neuronal migration in the adult brain and, particularly, in treatment of wound healing.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows morphological and immunocytochemical characterization of neural progenitors in culture. In Figures 1A and B, neural progenitors were isolated and purified from the subventricular zone of newborn rat brains and cultured on matrigel-coated coverslips in the presence of FGF-2 (20 ng/ml). Phase contrast images of neural progenitors at day 4 (Figure 1A) and day 6 (Figure 1B) are shown in culture. In Figure 1C, after the 6^{th} day in culture, the majority of cells are immunopositive for nestin, indicating that they are undifferentiated neural progenitors. In Figure 1D, BrdU incorporation (red) shows that the majority of cells are proliferating. The rare cells that are positive for the neuronal marker (TuJ, green, arrow) are nonproliferative. In Figures 1E and 1F, five days after the withdrawal of FGF-2, cells have differentiated into GFAP containing astrocytes (Figure 1E, red), Tuj positive neurons (Figure 1E, green) and GalC positive oligodendrocytes (Figure 1F, green). Cell nuclei were counterstained with Hoechst 33342 in Figures 1C, 1E and 1F. Scale bars, 80 μm in Figures 1A and 1B, 30 μm in Figure 1C; 19 μm in Figure 1D; 30 μm in Figures 1E and 1F.

Figure 2 demonstrates chemotaxis of neural progenitors stimulated by VEGF. Figure 2A is a schematic representation of a Dunn chamber (top view) with the overlying coverslip, showing the position of the inner well, bridge and outer well. In Figure 2B, cells over the annular bridge between the inner and outer wells of the chamber can be observed under phase-contrast optics. Cell migration was recorded continuously by time-lapse frame grabbing and the migration tracks were plotted in scatter diagrams (Figures 2C, 2D, 2E, and 2F). The starting point for each cell is at the intersection between the X and Y axes (0,0), and data points indicate the final positions of individual cells at the end of the 2-hour recording period. Chemotaxis was tested by placing 200 ng/ml of VEGF (Figure 2C) or FGF-2 (Figure 2E) in the outer well. The direction of the gradient is vertically upwards. Note that neural progenitors undergo chemotaxis and display a clear directionality of migration in the presence of a VEGF (Figure 2C), but not an FGF-2 (Figure 2E) gradient. For chemokinesis (Figures 2D and 2F), equal amounts of VEGF (20

ng/ml) or FGF-2 (20 ng/ml) were added in both inner and outer wells of the chamber. Arrow in Figure 2B indicates the direction of the outer well of the Dunn chamber. Scale bar, 50 μm.

Figure 3 shows migration tracks of neural progenitors. Figure 3A provides phase contrast photos showing a representative cell (*) migrating up a VEGF gradient. Arrow indicates the source of VEGF. Figure 3B shows migration tracks of 4 representative cells in the presence of a VEGF concentration gradient. The starting point for each cell is at the intersection between the X and Y axes (0, 0) and the source of VEGF is at the top. In Figure 3C, phase contrast photos show a neural progenitor randomly migrating in a uniform concentration of VEGF. Figure 3D shows migration tracks of 4 representative cells that migrate randomly under conditions of uniform VEGF distribution. The starting point for each cell is at the intersection between the X and Y axes (0, 0).

Figure 4 shows the migration speed (μm/hour) and forward migration index (FMI) values under different conditions. Cell migration speed was calculated for each time-lapse interval and the mean speed was derived for a period of 2 hours. Data are shown as mean ± SEM from at least 3 independent experiments. FMI values can be either positive or negative, depending on the direction in which the cells migrate. P is less than 0.01 by two-tailed unpaired t-test, which is significantly different from chemokinesis or an FGF-2 gradient.

Figure 5 shows VEGF receptor expression in neural progenitors. In Figure 5A, total cellular RNA was isolated and VEGF receptor mRNA expression was assessed by RNase protection analysis. Purified ^{32}P-labeled rat cRNA probes (probe) were hybridized to hybridization mix (probe + h.m.), yeast tRNA, or total RNA from cells grown in FGF-2 or starved of FGF-2 for 12 hours. Rat acidic ribosomal protein P0 was used as an internal control and the positive control was rat lung. In Figure 5B, quantitative analysis of VEGFR1 and VEGFR2 expression is shown in cells cultured in the presence of FGF-2 or starved of FGF-2 for 12 hours. P is less than 0.01 by two-tailed unpaired t-test, which is significantly different from cells in FGF-2 (n=3 experiments).

Figure 6 shows VEGF stimulated chemotaxis of neural progenitors through VEGFR2. In Figure 6A, diagrams show the migation patterns of neural progenitors under control conditions or in the presence of VEGF receptor blockers. Cells treated with the VEGFR2 blocking Ab (DC101) lost the chemotactic response to VEGF. In contrast, the VEGFR1 blocking Ab (MF1) did not affect progenitor migration. Figure 6B shows speed and FMI under different migration conditions. Figures 6C and 6D show migration tracks of representative cells (4 each condition) exposed to a VEGF concentration gradient, in the presence of either VEGFR2 blocking Ab (Figure 6C) or control (polysialic acid blocking) Ab (Figure 6D). The starting point for each cell is at the intersection between the X and Y axes (0, 0) and the source of VEGF is at the top in the gradient condition. P is less than 0.01 by two-tailed unpaired t-test, which is significantly different from DC101-treated cells.

Figure 7 shows FGF-2 enhanced ability of neural progenitors to chemotactically respond to a VEGF gradient. In Figure 7A, for a first group, FGF-2 was withdrawn at day 5 for 12 hours, then cells were exposed to a VEGF gradient. In Figure 7B, a second group was further cultured in the presence of FGF-2 after the 12-hour starvation period for 8 hours and then tested in a VEGF gradient In Figure 7C, the final positions of the cells after 2 hours of migration is indicated, with the starting point for each cell at (0, 0) and the source of VEGF (200 ng/ml) at the top. Figure 7D shows speed and FMI. Data are shown as mean ± SEM from 4 independent experiments. After 12 hours of FGF-2 starvation, cells lose their chemotactic response to the VEGF gradient. The starved neural progenitors resume their chemotactic response to VEGF upon re-addition of FGF-2 to the cultures for 8 hours (Figure 7C). Figure 7E shows VEGFR2 expression in neural progenitors cultured in FGF-2 or starved of FGF-2 for 12 hours. Western blot analysis was performed on immunoprecipitates with an anti-VEGFR2 Ab. P is less than 0.01 by two-tailed unpaired t-test.

Figure 8 shows the effect of VEGF on neural progenitors migrating from SVZ explants. Subventricular zone (SVZ) explants were cocultured with VEGF-secreting

C_2C_{12} cells and/or mock-transfected C_2C_{12} cells in collagen gel matrices in the presence (Figures 8A, 8B, 8D, 8E, and 8F) or absence (Figure 8C) of FGF-2. In Figure 8A, in the presence of FGF-2, neural progenitors migrate out of the SVZ explant in an asymmetric manner, with many more cells on the side of the VEGF-secreting C_2C_{12} cells than on the side of control C_2C_{12} cells. In Figure 8B, neural progenitors migrate out of the SVZ explant symmetrically when cultured with control C_2C_{12} cells on both sides. In Figure 8C, in the absence of FGF-2, few to no cells migrate out of the SVZ explant. In Figure 8D, a high power photograph shows the SVZ explant on the side of control C_2C_{12} cells. In Figure 8E, a high power photograph shows many neural progenitors migrating out of the SVZ explant toward VEGF-secreting C_2C_{12} cells. In Figure 8F, cells migrating out of the SVZ explant are positive for nestin, a marker for undifferentiated neural progenitors. Scale bar, 700 μm in Figures 8A, 8B and 8C; 100 μm in Figures 8D and 8E; 50 μm in Figure 8F.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, VEGF, a major angiogenic factor, which can be a guidance factor for endothelial progenitors and hematopoietic cells, is a chemoattractant for immature neural progenitors. Although FGF-2-stimulated progenitor cells express both VEGFR1 and VEGFR2, signaling through VEGFR2 appears to specifically mediate the effect of VEGF. Moreover, VEGFR2 expression, and consequently the capacity of cells to respond to gradients of VEGF, depends on the presence of FGF-2. Finally, migration of progenitors in a three-dimensional collagen matrix is directed by VEGF. This effect also requires the presence of FGF-2. The present invention thus provides a signaling mechanism that guides migrating neural progenitors in the central nervous system, a common guiding mechanism for neurogenic progenitors and endothelial cells.

Compositions of neural progenitors, VEGF, and/or FGF-2 are provided by the present invention. Such progenitor cells do not display antigenic markers (i.e., do not possess cell lineage-specific markers) for neuron-restricted or glia-restricted precursor

cells, including PSA-NCAM, doublecortin, NeuN, NG2 or A2B5. In addition, very few or none of the cells display differentiation markers such as tubulin, GFAP, O4 or Galc. However, most or substantially all of these cells are nestin-positive and negative for endothelial markers such as Von Willebrand factor and RECA-1. These compositions may also include one or more other suitable chemotactic or neurotrophic factors, such as growth factors (e.g., PDGF), netrins, semaphorins, ephrins, and Slits, for example. Antibodies to any of these markers can be used to isolate a suitable neural progenitor cell population.

Also provided by the present invention are methods of treating conditions associated with neurogenesis, and specifically, conditions associated with neuronal migration. A variety of clinical syndromes, including various forms of Lissencephalies, are related to deficient migration of neural cells. The consequences of these malformations include mental retardation, epilepsy, paralysis and blindness. In addition to inducing neurogenesis in early development, the present invention provides methods of inducing neuronal migration in the adult brain. Finally, the present invention provides methods of inducing cell migration central to wound repair. Such migration of neural progenitors and cell replacement may occur after administration of growth factors such as fibroblast growth factor 2 (FGF-2), as well as after transplantation of purified progenitors.

Immunocytochemical characterization reveals that progenitor cells maintained in the presence of FGF-2 do not display antigenic markers for neuron-restricted or glia-restricted precursor cells including PSA-NCAM, doublecortin, NeuN, NG2 or A2B5. Most remarkable is the absence of NCAM immunoreactivity in these cells. Very few cells displaying differentiation markers such as tubulin, GFAP, O4 or Galc and their presence is probably due to contamination of the initial cell population after isolation and purification of progenitors. On the other hand, the vast majority (98%) of cells were stained with an anti-nestin antibody. Importantly, nestin-positive cells were negative for endothelial markers such as Von Willebrand factor and RECA-1. Together, this indicates that the

progenitor or stem cells do not yet possess cell lineage-specific markers for neurons or glial cells.

It should be emphasized here that progenitor cells in the presence of FGF-2 do not generate neurospheres but rather they spread out evenly and form a monolayer. This may expose cells to FGF-2 more evenly and favor the formation of homogenous population of undifferentiated progenitors as has previously been suggested (Wu et al., 2002). Since more than 96% of these progenitor cells displayed immunoreactivity for neuronal and glial markers after differentiation, these cultures are composed essentially of multipotential neural progenitors or stem cells.

The Dunn chemotaxis chamber shows directly that concentration gradients of VEGF induce directional sensing by neural progenitor cells. Under basal conditions in the presence of uniform concentrations of FGF-2, progenitor cells were polarized and motile and moved randomly across a two-dimensional substrate with a mean speed of about 58 μm/hour (Figure 4A). When cells were exposed to linear gradients of VEGF, their leading processes became stable over a substantial portion of the time-course, and the cells continued to migrate up the VEGF gradient. In contrast, when cells were exposed to uniform concentrations of VEGF, no biased displacement was observed, and the mean displacement speed was not significantly different from that measured in the presence of FGF-2 alone. Thus, although VEGF specifically affects the direction of progenitor migration, it does not appear to stimulate migration rate per se. Similar results were obtained with VEGF-$C_{\Delta N \Delta C}$. VEGF-$C_{\Delta N \Delta C}$ is the proteolytically processed form of VEGF-C (21 kDa, which binds to and activates VEGFRs -2 and –3 (Joukov et al., 1997). We also demonstrated that the effect of VEGF and VEGF-$C_{\Delta N \Delta C}$ was specific, since concentration gradients of FGF-2 did not induce directional migration of neural progenitor cells. In line with these observations, experiments with SVZ explants illustrate that FGF-2-stimulated progenitors are invasive in a three-dimensional collagen matrix in which they migrate towards the source of VEGF.

FGF-2-stimulated progenitors express the two tyrosine kinase receptors for VEGF, namely VEGFR1 and VEGFR2, but not VEGFR3. These findings confirm previous observations demonstrating that members of the VEGF receptor family are expressed not only by blood and endothelial cells, but also by immature neurons from different regions of the nervous system (Carmeliet and Storkebaum, 2002; Jin et al., 2002; Louissaint et al., 2002; Ogunshola et al., 2002; Svensson et al., 2002). Similar to endothelial cells, FGF-2 is a critical regulatory factor for VEGF receptor expression in progenitor cells. The receptor is expressed in the presence of FGF-2, and withdrawal of the growth factor leads to a significant downregulation, as measured by RNase protection assay and Western blotting. Migratory responses to VEGF also require the presence of FGF-2 in the two-dimensional as well as three-dimensional migration models.

It is unlikely that downregulation of VEGF receptor expression and the lack of chemotactic responses are due to death or suffering of cells in the absence of FGF-2, which is demonstrated by the following: 1) After removal of FGF-2 for 12 hours, cells maintained in neurobasal medium supplemented with B27 displayed no difference in morphology compared to control cultures; 2) Hoechst 33258 staining of cell nuclei did not reveal any difference between cultures kept in the presence or absence of FGF-2; 3) Video analysis revealed that cells in the absence the FGF-2 exhibited random migration with the same migration speed as control cells in the presence of FGF-2; 4) FGF-2 starvation did not change the expression of acidic ribosomal phosphoprotein (P0). In vitro, FGF-2 is known to stimulate mitotic activity in progenitors cells and to maintain these cells in an undifferentiated state (Palmer et al., 1997; Tropepe et al., 1999). Since withdrawal of FGF-2 from cultures is a standard procedure used to induce the differentiation of FGF-2-stimulated progenitors (Palmer et al., 1997; Tropepe et al., 1999), the more differentiated progenitors may loose VEGFR expression as well as the capacity to respond to VEGF. However, the effect of FGF-2 withdrawal was reversible upon the re-application of FGF-2 to the medium after 8 hours. VEGF receptor expression may also be induced by FGF-2 in differentiated neurons.

Although FGF-2-stimulated progenitors express both VEGFR1 and VEGFR2, only the latter appears to be required for directional migration in response to VEGF. Blocking receptor function with a specific monoclonal Ab completely blocked the migratory response to VEGF. In contrast, function-blocking antibodies against VEGFR1 had no effect. These results demonstrate that VEGFR2 signaling, previously shown to regulate survival and proliferation of neuronal progenitors (Carmeliet and Storkebaum, 2002; Jin et al., 2002; Ogunshola et al., 2002; Svensson et al., 2002), is also involved in the migration of these cells. The respective roles of VEGFR1 and VEGFR2 in regulating cell migration are not well understood. While, similar to FGF-2-stimulated neural progenitors, the VEGFR2 pathways appears to be involved in the migration of endothelial cells (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001), in other cells such as a neuroectodermal cell line from a human cerebellar tumor, and monocytes, VEGFR1 signaling dominates (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Bagnard et al., 2001; Forstreuter et al., 2002).

VEGF signaling may also be involved specifically in directing migration of multipotent neural progenitors rather than in guiding neuron- or glia-restricted precursors. In one embodiment, VEGF signaling plays a role in the initial phase of neurogenesis, in particular in the formation of neurogenic matrix or neurogenic niches in association with angiogenic sites. Indeed, high levels of VEGF transcripts were detected in the neurogenic matrix of the ventricular and subventricular zones in the embryonic as well as in the postnatal rat brain (Breier et al., 1992). Moreover, receptors for VEGF are expressed in neuroproliferative zones in vivo (Jin et al., 2002; Breier et al., 1995).

Clusters of proliferating cells in the dentate gyrus of the adult mammalian brain have been shown to contain dividing endothelial as well as neural progenitors and proliferative clusters have been associated with growing capillaries. Some of the cells in the clusters were immunoreactive for VEGFR2, while VEGF immunoreactivity was seen in tissue surrounding the clusters.

Neurogenesis and angiogenesis, however, occur concurrently in the adult avian brain (Louissaint et al., 2002). In this system, immature neurons generated in the subventricular germinal zone migrate to reach active angiogenic spots in the adjacent tissue. Studies have shown the association of neural progenitors with blood vessels (Capela and Temple, 2002). Neural and angiogenic progenitors may be recruited to form proliferative niches either by common signaling mechanisms or by cues acting simultaneously, VEGF, previously described as a chemoattractant for endothelial progenitors and blood cells (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001), is also able to direct neural progenitor migration.

Thus, VEGF could be a common guidance cue for recruiting neural progenitors and endothelial cells to correct sites for differentiation. Such a role is consistent with the observation that VEGF promotes the survival of both immature neurons and endothelial cells (Sondell et al., 1999; Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001), stimulates the proliferation of both cells types (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Ogunshola et al., 2002), and protects them from injury in vitro (Jin et al., 2000; Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Matsuzaki et al, 2001) as well as in vivo (Matsumoto and Claesson-Welsh, 2001; Robinson and Stringer, 2001; Ogunshola et al., 2002). The coordinated regulation of neurogenesis and angiogenesis by VEGF highlights the importance of this signaling pathway in the morphogenesis of nervous tissue.

The present invention also provides for repair after brain injury. Increasing evidence indicates that VEGF and its receptors are activated after various lesions in the brain including stroke (Carmeliet and Storkebaum, 2002). For example, focal or global brain ischemia induces an acute upregulation of VEGF expression that is consistent with the notion that the promoter region of VEGF contains hypoxia-responsive elements (Carmeliet and Storkebaum, 2002; Zhang and Chopp, 2002; Sun et al., 2003). In addition, several cytokines and growth factors known to be associated with ischemia, upregulate VEGF expression in many cell types. VEGF expression under these conditions appears to

be important for initiating neovascularization and regenerating capillaries in the damaged zone. Injuries to the brain, including ischemia and seizure, also stimulate the mitotic activity of neural progenitors in neurogenic centers, and enhance neurogenesis (Parent et al., 2002; Parent and Lowenstein, 2002; Iwai et al., 2003; Kokaia and Lindvall, 2003). VEGF under these circumstances could be an attractant guidance factor for neural progenitors, just as it is for endothelial progenitors and blood cells. Thus VEGF, along with FGF-2, can be therapeutic molecular tools, with efficacy in directing the migration of endogenous or transplanted progenitors to injured regions of the brain.

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EXAMPLES

Materials and Methods

Isolation and cultures of neural progenitors

All animal experiments were conducted in accordance with Swiss laws, previously approved by the Geneva Cantonal Vetirinary Authority. The SVZ was dissected from coronal slices of newborn rat brains, dissociated mechanically and trypsinized as described (Lim et al., 2000). SVZ progenitors were purified using percoll gradient centrifugation as described (Lim et al., 2000) and seeded onto matrigel (0.24 mg/cm^2)- or laminin-coated coverslips. Isolated cells were allowed to grow in Neurobasal medium supplemented with 20 ng/ml FGF-2 (human recombinant, kindly provided by Dr P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy), 1 × B27, 2 mM glutamate, 1 mM sodium pyruvate, 2 mM N-acetyl-cysteine, and 1% penicillin-streptomycin. Cultures were fed every three days with fresh medium containing 20 ng/ml FGF-2.

Immunocytochemistry

Immunostaining of cultures was performed as previously described (Wang et al., 1996; Vutskits et al., 2001). The following primary antibodies and dilutions were used: mouse monoclonal antibody (mAb) against nestin (Biogenesis, UK, 1:300 dilution); mouse mAb against A2B5 (Eisenbarth et al., 1979) (hybridoma supernatant, ATCC, Rockville, MD, 1:5 dilution); Men B (Meningococcus group B) mouse IgM mAb (1:500 dilution) that specifically recognizes α 2-8-linked PSA with chain length superior to 12 residues (Rougon et al., 1986); anti-GalC (Ranscht et al., 1982) mouse IgM mAb (culture supernatant, 1:5 dilution); Tuj mouse mAb directed against β-tubulin isotype III (1:400 dilution) (Sigma, Saint Louis, Missouri); a rabbit polyclonal Ab to GFAP (Dakopatts, Copenhagen, Denmark, 1:200 dilution); a rabbit polyclonal Ab against NG2 (Chemicon International, California, 1:400 dilution); a goat polyclonal Ab against Doublecortin (Santa Cruz Biotecnology, 1:300 dilution); a mouse mAb against Neu N (Chemicon International, California, 1:100 dilution). The rabbit antiserum directed against the NCAM protein core was a site-directed Ab recognizing the seven NH-2-terminal residues

of NCAM (1:1000 dilution) (Rougon and Marshak, 1986). O4 monoclonal Ab (hybridoma supernatant, 1:5 dilution) (Eisenbarth *et al.*, 1979) was used to identify undifferentiated oligodendrocytes Hoechst 33258 was used to counterstain cell nuclei in some cases. Fluorescence was examined with a fluorescence microscope (Axiophot; zeiss, Oberlochen, Germany). Controls treated with non-specific mouse IgM, or IgG preimmune sera or secondary Abs alone showed no staining. In double immunolabeling experiments, the use of only one primary Ab followed by the addition of both anti-mouse FITC and anti-rabbit TRITC-conjugated secondary Abs resulted only in single labeling. Proliferating cells were identified with a monoclonal Ab against BrdU (Boehringer, 1:50 dilution) after 20-hour incorporation.

Migration assays using Dunn chamber

Chemotaxis of neural progenitors was directly viewed and recorded in stable concentration gradients of VEGF (human recombinant, 165-amino acid homodimeric form, purchased from Peprotec Inc, Rochy Hill, NJ) using the Dunn chemotaxis chamber (Weber Scientific international Ltd, Teddington, UK) (Zicha et al., 1991; Allen et al., 1998). (Recombinant human VEGF-$C_{\Delta N \Delta C}$ (kindly provided by Dr. M. Skobe, Cancer Center, Mount Sinai Medical Center, New York) was used in some experiments.) This device is made from a Helber bacteria counting chamber by grinding a circular well in the central platform to leave a 1mm wide annular bridge between the inner and the outer well. Chemoattractants added to the outer well of the device will diffuse across the bridge to the inner blind well of the chamber and form a gradient. This apparatus allows one to determine the direction of migration in relation to the direction of the gradient. Coverslips with cells were inverted onto the chamber and cell migration was recorded through the annular bridge between the concentric inner and outer wells, and a period of 2 hours was chosen to assess cell migration. In these studies, a systematic sampling was applied and all cells within the migration region of the chamber were recorded and analyzed. Data were recorded every 10 minutes using a ZEISS 10 × objective via a HAMAMATSU CCD video camera using Openlab software. In these chemotaxis experiments, the outer well of the

Dunn chamber was filled with medium containing 200 ng/ml VEGF and 20 ng/ml FGF-2 and the concentric inner well with only medium and FGF-2. For chemokinesis experiments, VEGF (20 ng/ml) or FGF-2 (20 ng/ml) was added to both outer and inner wells of the Dunn chamber. MF1, a VEGFR1 blocking Ab, DC101, a VEGFR2 blocking Ab (both added at 20 μg/ml, and kindly provided by Dr. D. Hicklin, ImClone Systems Incorporated, New York), were used to block the function of the corresponding VEGF receptor. A polysialic acid blocking Ab was used as a control.

Directionality of cell movement was analyzed using scatter diagrams of cell displacement. The diagrams were oriented so that the position of the outer well of the chamber was vertically upwards (y direction). Each point represents the final positions of the cells at the end of the recording period where the starting point of migration is fixed at the intersection of the two axes.

To determine the efficiency of forward migration during the 2-hour recording period, the FMI was calculated as the ratio of forward progress (net distance the cell progressed in the direction of VEGF source) to the total path length (total distance the cell traveled through the field) (Foxman et al., 1999). FMI values were negative when cells moved away from the source of VEGF. The cell speed was calculated for each lapse interval recorded during the 2-hour period.

RNA Purification and RNase Protection Assay

Neural progenitors at 6 days of culture in FGF-2 or after starvation of FGF-2 for 12 hours were used for RNA preparation. Total cellular RNA was purified using Trizol reagent (Invitrogen). RNase protection assays were performed using cRNA probes for rat VEGFR1 and VEGFR2 as described in Pepper et al. (2000).

Immunoprecipitation and Western Blotting

Neural progenitors from the normal cultures in FGF-2 or from cultures starved of FGF-2 for 12 hours were lysed and VEGFR2 protein was immunoprecipitated from cell lysates with a polyclonal Ab (sc-504; Santa Cruz Biochemicals, Santa Cruz, CA) recognizing amino aids 1158 to 1345 in the mouse VEGFR2 carboxy terminus. Western

blot was performed with a polyclonal anti-VEGFR2 Ab (sc-315; Santa Cruz Biochemicals) recognizing the mouse carboxy terminal amino acids 1348 to 1367.

SVZ explants cultures

The frontal lobes of the brains of one-day-old Sprague-Dawley rat pups (Sizv, Zurich, Switzerland) were isolated and cut into 300 μm thick coronal sections with a McIllwain tissue chopper. From these slices the anterior part of the subventricular zone (SVZ) was microdissected. The SVZ explants were embedded in a collagen matrix and cultured for 7 days in chemically-defined serum-free medium (50% Dulbecco's modified Eagle's medium [Gibco, Berlin, Germay], 50% F12, HEPES, Tris–HCl, and complemented with transferrin human 20 μg/ml, putrescine 100 μM, sodium selenite 30 nM, triiodothyronin 1 nM, docosahexaenoic acid 0.5 μg/ml, arachidonic acid 1 μg/ml, insulin 60 U/l) under 5% CO_2. The medium was changed every 3rd day. For co-culture experiments, SVZ explants were cultured in the presence of murine C_2C_{12} myoblasts that had been engineered to secret VEGF (Rinsch et al., 2001). C_2C_{12} cells were suspended in a drop of collagen matrix which was placed at a distance of approximately 1,000 μm from the SVZ explant. As a control, mock-transfected cells of the same origin were placed into the collagen matrix in a similar manner and at the same distance, but on the opposite side of the explant.

Cell migration was assessed at the end of the 7th day in culture. Three categories were established: 1, no migration: no or only a few cells emigrated from the explants; 2, symmetrical migration: numerous cells had left the explants, the distance of the migrating front of the cells exceeded 50 μm, no directionality of migration; 3, asymmetrical or directional migration: when the distance of the migrating front were at least twice that on the other side and exceeded 50 μm.

Example 1: FGF-2 Stimulated Neural Progenitors

To investigate the potential role of VEGF in the regulation of FGF-2-stimulated neural progenitor migration, SVZ cells were isolated from newborn rats and cultured in the presence of FGF-2 in defined medium as described (Lim et al., 2000). Four days after

plating, the cells had an immature, round or bipolar morphology (Figure 1A). Daily observations indicated that cells divided, formed loose colonies and by day 6 had formed a monolayer (Figure 1B). At this stage, the vast majority (98%) of cells were stained with an anti-nestin antibody (Ab) (Figure 1C), which is considered to be a marker of neural progenitors. Less than 3.2% of the cells expressed the neuronal marker Tuj, PSA-NCAM and BrdU incorporation showed that these cells did not divide (Figure 1D). Very few to no cells displayed immunoreactivity for GFAP or Gal C, markers for astrocytes and oligodendrocytes respectively (not shown). With the exception of the few differentiated cells, progenitor cells maintained in the presence of FGF-2 did not display antigenic markers for neuron- or glia-restricted precursor cells including PSA-NCAM, doublecortin, NeuN, NG2 or A2B5 (not shown). In addition, nestin-positive cells were negative for endothelial markers such as Von Willebrand factor and RECA-1 (not shown). These results indicate that the cultures consist of immature cells that do not yet possess cell lineage-specific markers for neurons or glial cells. When cultures were allowed to differentiate under conditions shown previously to stimulate both neuronal and glial differentiation (Palmer et al., 1997), more than 96% of the population displayed immunoreactivity for neuronal and astrocytic markers (Tuj+, 21%; GFAP+, 75%) (Figure 1E). The remaining population was immunoreactive for oligodendrocyte markers A2B5 or Gal C (Figure 1F). These observations concur with earlier studies (Tropepe et al., 1999) showing that FGF-2-expanded cells are multipotential neural progenitors that can give rise to neurons, astrocytes and oligodendrocytes, the three major cell types in central nervous system.

Example 2: Chemoattraction of FGF-2-stimulated progenitors by VEGF

The response of neural progenitors to gradients of VEGF in the direct-viewing Dunn chemotaxis chamber (Zicha et al., 1991; Allen et al., 1998) was examined. This apparatus allows for direct monitoring of cell locomotion including analysis of migration speed, turning behavior and directionality (see Materials and Methods). It has been established that chemoattractants added to the outer well of the Dunn chamber diffuse

across the bridge to the inner well (Figure 2A, 2B) and form a linear steady gradient within about 30 min of setting up the chamber (Zicha et al., 1991; Webb et al., 1996). The gradient remains stable for about 30 hours thereafter (Zicha et al., 1991; Webb et al., 1996). To study the chemotaxis of neural progenitors, the outer well of the Dunn chamber was filled with medium containing different concentrations of VEGF and the concentric inner well with medium only. Coverslips with progenitor cells were inverted onto the chamber and cell locomotion (Figure 2) was recorded over one part of the bridge region. It was observed that progenitors at day 6 maintained in the presence of FGF-2 and exposed to concentration gradients established with 200 ng/ml VEGF, displayed strong positive chemotaxis (Figure 2C). The scatter diagram of cell displacements in Figure 2C demonstrates a strong directional bias of migration toward the source of VEGF. In contrast, when VEGF was added to both inner and outer wells (chemokinesis conditions), cells remained motile but the population as a whole showed no clear preference for displacement (Figure 2D). In these experiments, FGF-2 (20 ng/ml) was systematically included in the medium during the recording of neural progenitor chemotaxis or chemokinesis. FGF-2, however, had no chemotactic effect on these cells, irrespective of whether or not VEGF was present (Figure 2E, 2F). No difference was detected in migratory behavior between cells exposed to an FGF-2 gradient (Figure 2E) and cells exposed to a uniform concentration of FGF-2 (Figure 2F).

These observations were confirmed by the examination of individual cell tracks. As shown in Figure 3, progenitors exposed to a VEGF gradient migrated efficiently towards the source of VEGF (Figure 3A, 3B), while those under conditions of chemokinesis (Figure 3C, 3D) or exposed to an FGF-2 gradient (not shown) made random turns during migration.

To measure the efficiency of directed cell migration, each cell's forward migration index (FMI), i.e. the ratio of the most direct distance the cell progressed towards the gradient source (the outer well of the Dunn chamber) over its total path length, was calculated. These quantitative analyses revealed that both the migration speed (Figure 4A)

and the forward migration index (Figure 4B) of cells exposed to VEGF in the presence of FGF-2 were significantly greater than those of cells exposed to an FGF-2 gradient or to a uniform concentration of VEGF or FGF-2 (chemokinesis). The attractive effect of VEGF was similar on laminin-, poly-L-lysine- or matrigel-coated coverlips. These data indicate that VEGF is attractant for FGF-2-stimulated neural progenitors and that this effect is matrix- independent.

Example 3: VEGF receptor expression in progenitors

Signaling receptors for VEGF include VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), both of which belong to the receptor tyrosine kinase superfamily (Matsumoto and Claesson-Welsh, 2001). To determine whether these receptors are expressed by FGF-2-stimulated progenitors, total cellular RNA was analyzed by RNase protection assay. This revealed that the cells express VEGFR1 and VEGFR2 when cultured in the presence of FGF-2. mRNA for VEGFR-3 was not detected in these cultures (Figure 5A). Previous studies have indicated that in endothelial cells, FGF-2 regulates expression of VEGF receptors. To explore whether FGF-2 affects the expression of these receptors in FGF-2-stimulated progenitors, mRNAs for VEGFR1 and VEGFR2 were analyzed after 12-hour starvation of FGF-2. Withdrawal of FGF-2 led to a marked, 5-fold decrease in the level of VEGFR1 and VEGFR2 transcripts (Figure 5A, 5B). These results demonstrate that FGF-2-stimulated progenitors express mRNA for both VEGFR1, VEGFR2 and that FGF-2 is required for this expression.

Example 4: VEGF-induced chemotaxis is mediated through VEGFR2

To identify the receptor(s) involved in VEGF-induced chemotaxis, the migratory behavior of cells toward VEGF in the presence of functional blockers (neutralizing Abs) of VEGFR1 or VEGFR2 was analyzed. As shown in Figure 6, the chemotactic response of cells to VEGF was completely abrogated by the VEGFR2 blocking Ab DC101 (Figure 6A, 6C). In contrast, the VEGFR1 blocking Ab MF1 did not affect chemotaxis (Figure 6A). These observations were confirmed by measurements of speed and forward migration index (Figure 6B). In the absence of a VEGF gradient, addition of anti-

VEGFR2 had no significant effect on progenitor migration. These experiments demonstrate that VEGF stimulates chemotaxis of progenitor cells through VEGFR2. This conclusion received further support from experiments in which concentration gradients of VEGF-$C_{\Delta N \Delta C}$ was used to induce chemotaxis. It was observed that VEGF-$C_{\Delta N \Delta C}$ could efficiently induce chemotaxis of progenitor cells and that this effect was prevented by the VEGFR2 blocking Ab (data not shown). Furthermore, since VEGF-$C_{\Delta N \Delta C}$ exerts its function through VEGFR2 and VEGFR3, and since VEGFR3 is not expressed by FGF-2-stimulated neural progenitors, these results strengthen the conclusion that signaling through VEGFR2 mediates chemoattraction of progenitor cells by VEGF.

Example 5: FGF-2 is required for VEGF to stimulate chemotaxis of progenitors

Inasmuch as FGF-2 is required for the expression of VEGFR2, the chemotactic response of progenitor cells to VEGF may also be dependent on the presence of FGF-2 and, thus, the migratory response of progenitors to VEGF in the absence of FGF-2 was examined. Cells at 5 days of culture were starved of FGF-2 for 12 hours and then exposed to a VEGF gradient (Figure 7A). As shown in Figure 7B, starved cells failed to undergo chemotaxis in response to VEGF. Cells migrated randomly in a manner similar to when they were exposed to a uniform concentration of VEGF. In agreement with these results, and confirming the data of the RNase protection assay (Figure 5), Western blot analysis revealed little to no expression of VEGFR2 protein in the absence of FGF-2, while substantial expression was detected in the presence of FGF-2 (Figure 7E).

To determine whether the effect of FGF-2 withdrawal is reversible and whether cells could chemotactically respond to VEGF upon re-addition of FGF-2 to the cultures, FGF-2 was included in the medium after a 12-hour starvation period and the cells were further cultured for 8 hours. Diagrams of displacements of motile cells (Figure 7C) and a quantitative analysis of forward migration index and speed (Figure 7D) demonstrated that the loss of chemotaxis was rescued after an 8-hour re-incubation with FGF-2. Taken together, these data demonstrate that FGF-2 is necessary for the expression of VEGFR2

and for an adequate migratory response of progenitors to concentration gradients of VEGF.

Example 6: VEGF affects migration of progenitors from subventricular zone

To determine whether the effect of VEGF on FGF-2-stimulated progenitor migration is functionally relevant, the effect of VEGF on cell migration from SVZ explants was investigated. SVZ explants were co-cultured in a three-dimensional collagen matrix with aggregates of control or VEGF-secreting murine C_2C_{12} myoblasts, in the presence or absence of FGF-2. When explants were co-cultured with aggregates of mock-transfected cells in the presence of FGF-2 (20 ng/ml) (Fig 8B, 8D), migrating cells were symmetrically distributed around the explants (10/10 explants). When SVZ explants were co-cultured, in the presence of FGF-2, with VEGF-expressing cells placed on one side and with mock-transfected cells on the other, cell migration was highly asymmetric (Fig 8A, 8E) (10/20 explants with cells migrating predominantly towards VEGF-secreting C_2C_{12} cells, and, 10/20 explants with a symmetric migratory pattern). In contrast, when explants were co-cultured with control or VEGF-expressing cells in the absence of FGF-2, no significant cell migration from SVZ explants was observed (Figure 8C) (10/10 explants). Similar results were obtained after application of VEGF in the absence of FGF-2 (4/4 explants). The application of VEGF and FGF-2 together or FGF-2 alone resulted in symmetric migration (12/12). To determine whether cells migrating in response to VEGF are immature progenitors, immunocytochemical staining with an anti-nestin Ab was carried out. Migrating cells stained positively for nestin (Figure 8F) and were negative for PSA-NCAM (a marker for immature neurons, not shown), confirming that they were indeed immature progenitor cells. Together, these results indicate that immature progenitor cells migrate in response to VEGF gradients, and that FGF-2 is required for this effect.

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Figure 1

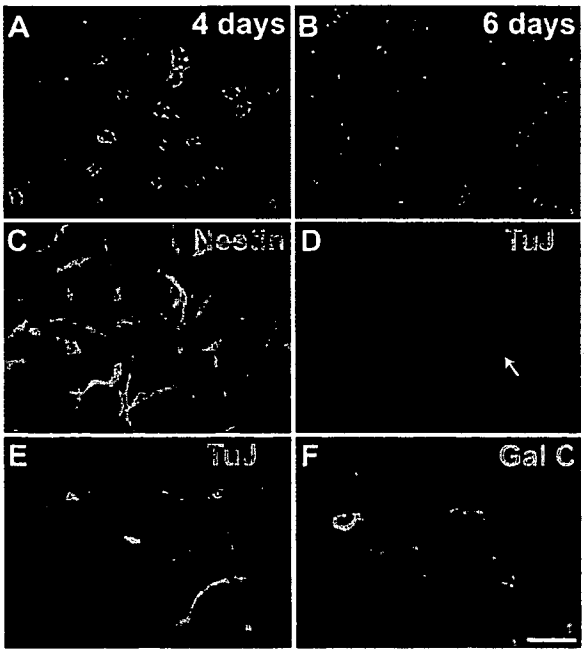

Figure 2

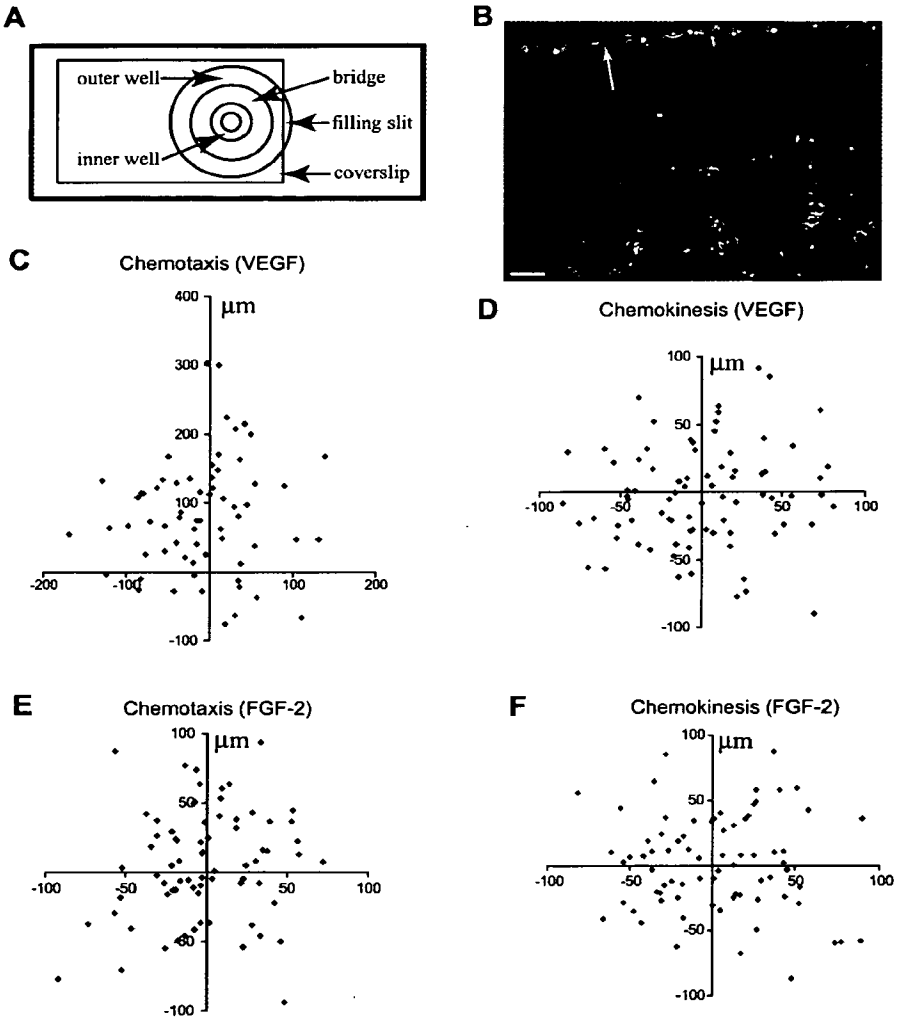
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Figure 3

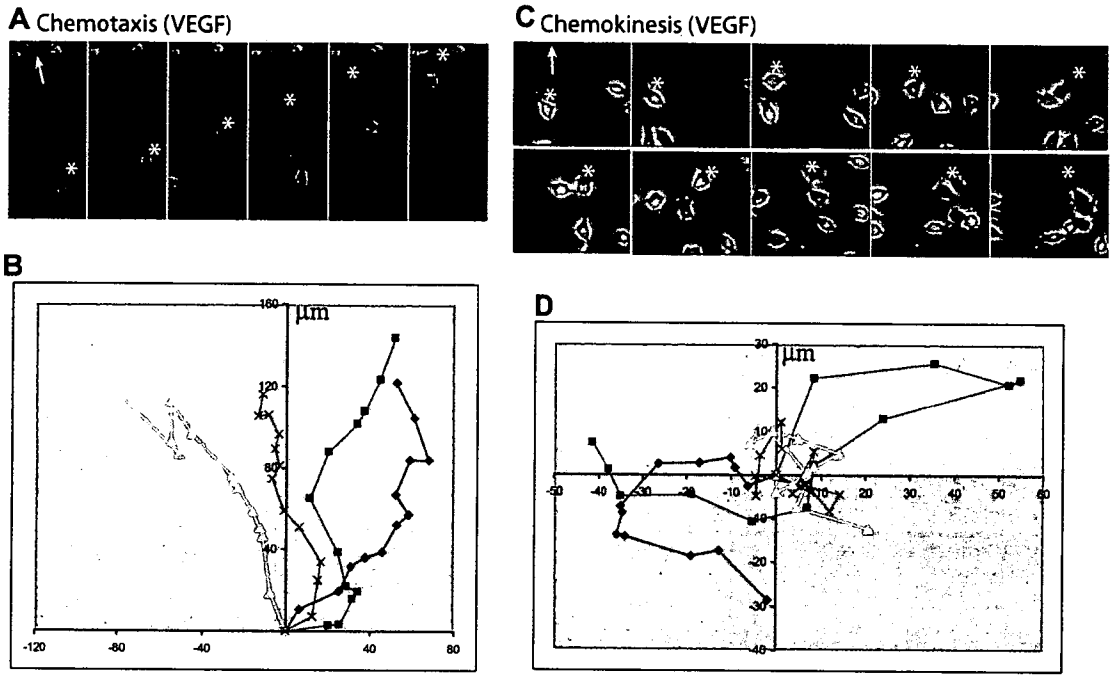

Figure 4

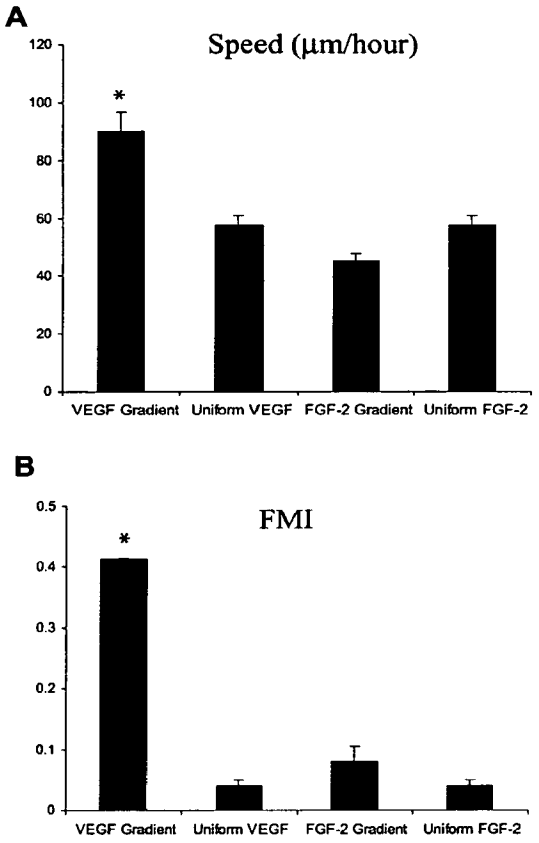
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Figure 5

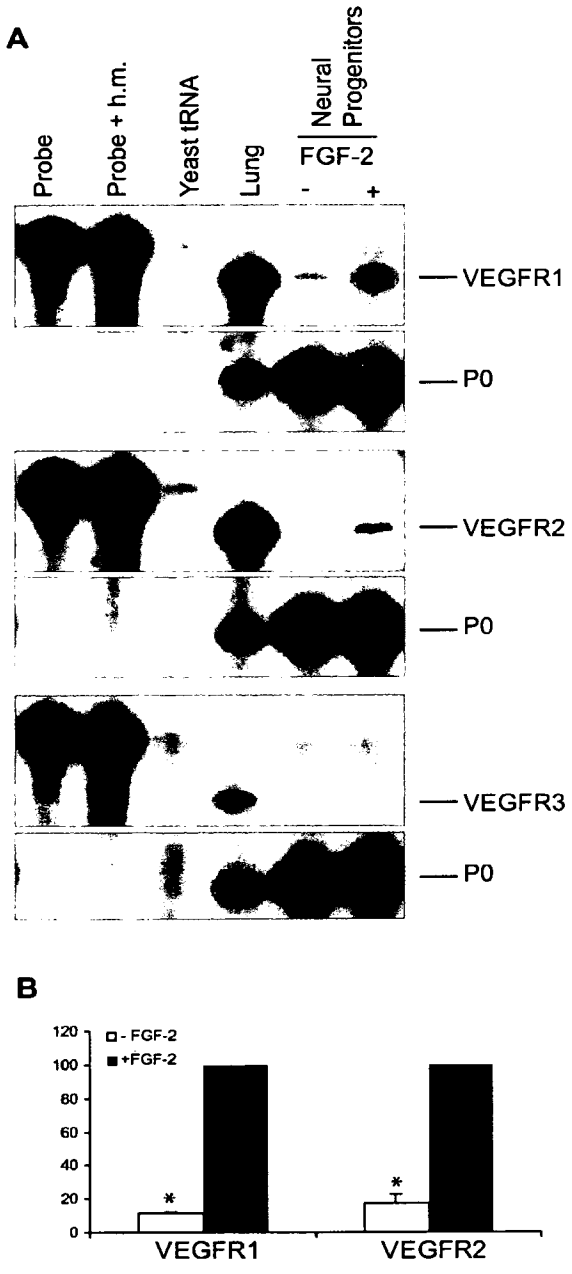
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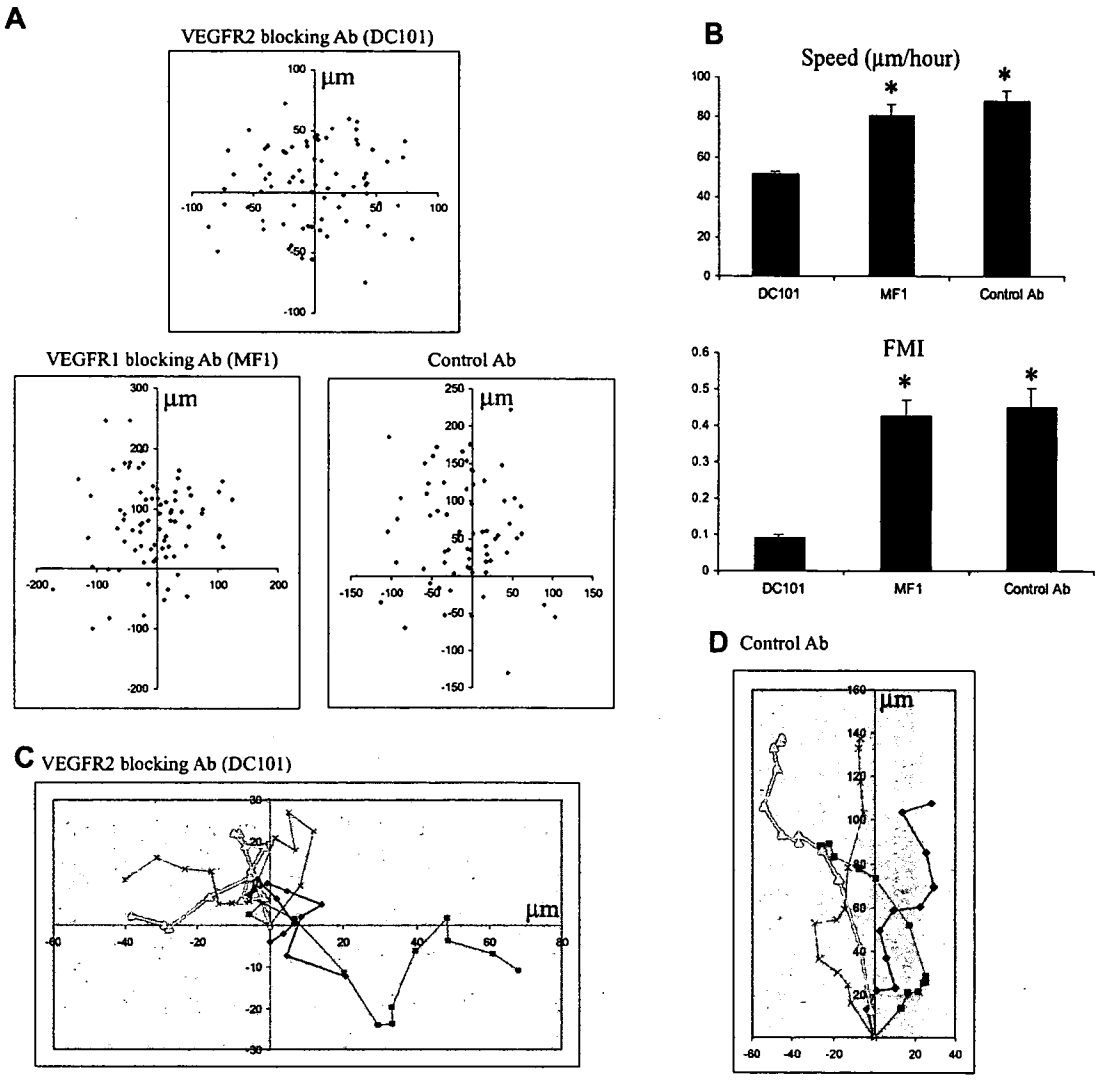
Figure 6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Speed (µm/hour)
DC101
MF1
Control Ab
FMI
C VEGFR2 blocking Ab (DC101)
D Control Ab

Figure 7

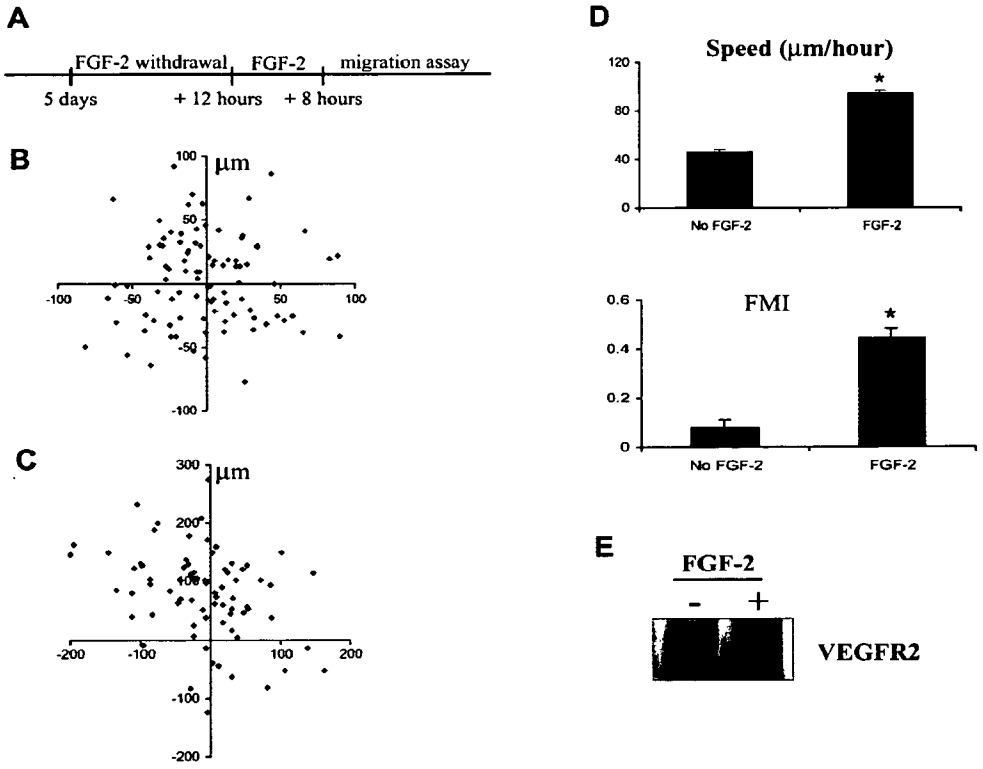
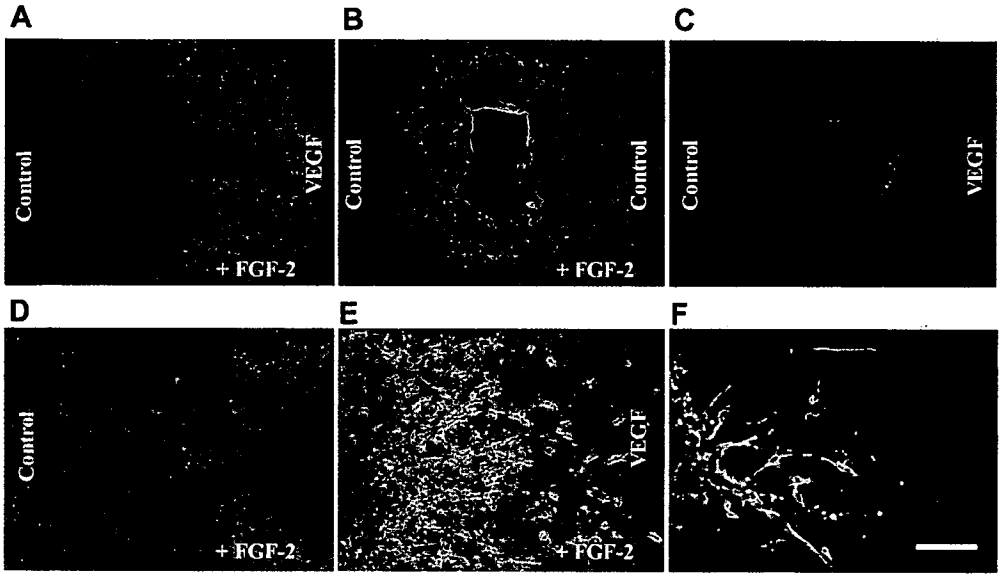

A
FGF-2 withdrawal
FGF-2
migration assay
5 days
+ 12 hours
+ 8 hours
B
µm
100
50
-50
-100
-100
-50
50
100
C
µm
300
200
100
-100
-200
-200
-100
100
200
D
Speed (µm/hour)
120
80
40
0
No FGF-2
FGF-2
FMI
0.6
0.4
0.2
0
No FGF-2
FGF-2
E
FGF-2
-
+
VEGFR2

Figure 8


A
B
C
Control
VEGF
+ FGF-2
Control
Control
+ FGF-2
Control
VEGF
D
E
F
Control
+ FGF-2
VEGF
+ FGF-2